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Published
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(54) Title: SUPERANTIGEN INDUCED IMMUNE NON-RESPONSIVENESS

(57) Abstract

Staphylococcal enterotoxin based compounds are provided for immunosuppression. The compounds may be used to reduce T-cell response to antigens, based upon the particular variable regions of the T-cell receptor. The subject compounds find use in protecting against an immune response.

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SUPERANTIGEN INDUCED IMMUNE NON-RESPONSIVENESS

INTRODUCTION

Technical Field

The field of this invention is immunomodulation.

Background

There are many reasons for wishing to modulate an immune response. While the immune system polices for the presence of foreign antigens, as a result of infection, tumors, or the like, in many situations there is a need to prevent an immune response. These situations vary from transplantation, to autoimmune diseases, allergic responses, and the like. In such situations, the inhibition may vary from a relatively short term to a relatively long term. For the most part, immunosuppressive agents today tend to suppress the entire system, where compounds such as cyclosporin or FK506 are employed. The suppression of the entire immune system exposes the individual to high susceptibility to pathogenic infection.

There have been many reports of naturally occurring immunosuppressive agents. Many pathogens are reported to have polysaccharide or glycoprotein which serve to reduce the ability of the infected host to respond to the pathogen. Viruses, such as HIV, are able to induce immunosuppression, reportedly based on inactivating helper T-cells. Antibodies to T-cell receptors are reported to reduce immune responsiveness.

There is substantial interest in being able to develop agents which can provide some level of specificity in modulating or reducing the immune response.

Relevant Literature

Staphylococcal enterotoxin and Mls are members of a family of antigens termed superantigens. Carlsson et al., J. Immunol. 140: 2484-2488 (1988) and Peavy et al., ibid. 105: 1453-1458 (1970); Festenstein, Transplant Rev. 15: 62-88 (1973); Janeway et al., Immunol. Rev. 107: 61-88 (1989). These compounds are found to stimulate powerful polyclonal proliferative responses of murine and human T lymphocytes bearing particular T-cell antigen receptor Vβ sequences. White et al., Cell 56: 27-35 (1989) and Kappler et al., Science 244: 811-813 (1989). Superantigens have also been reported to induce T-cell non-responsiveness by clonal deletion or functional inactivation of T-cells. Kappler et al., supra and Rammensee et al., 339: 541-544 (1989). Peptide specific T anergy induced in vitro is described in Lamb et al., J. Exp. Med. 157: 397-405 (1983) and Jenkins and Schwartz, J. Exp. Med. 165: 302-309 (1987). Cloned human CD4+ T-cells specific for residues 307-319 of the carboxyl terminus of influenza virus HA and restricted by HLA-DR1 is described by Lamb et al., Nature 399: 66-69 (1982) and Rothbard et al., Cell 52: 515-523 (1988).

SUMMARY OF THE INVENTION

Immune non-responsiveness is achieved by administering an effective amount of at least a fragment of a superantigen, such as Staphylococcal enterotoxins to a lymphoid system comprising B and T-cells.

DESCRIPTION OF THE DRAWINGS

In accordance with the subject invention, superantigens, fragments or derivatives thereof, particularly Staphylococcal enterotoxins, are used to modulate the immune response. By administering a sufficient amount of a at least a fragment of the Staphylococcal enterotoxin, which is active in binding to an appropriate receptor, immune non-responsiveness

can be achieved. The receptor may be any receptor associated with the immune system, e.g. cytosine receptor T-cell receptor, etc. Particularly, the enterotoxin associated with specific Vβ subunits of T-cell receptors can provide for clonal anergy of T-cells which have the subunit which binds to the enterotoxin fragment. In this way, only a portion of the T-cells in a complete T and B cell system derived from a host may be made non-responsive.

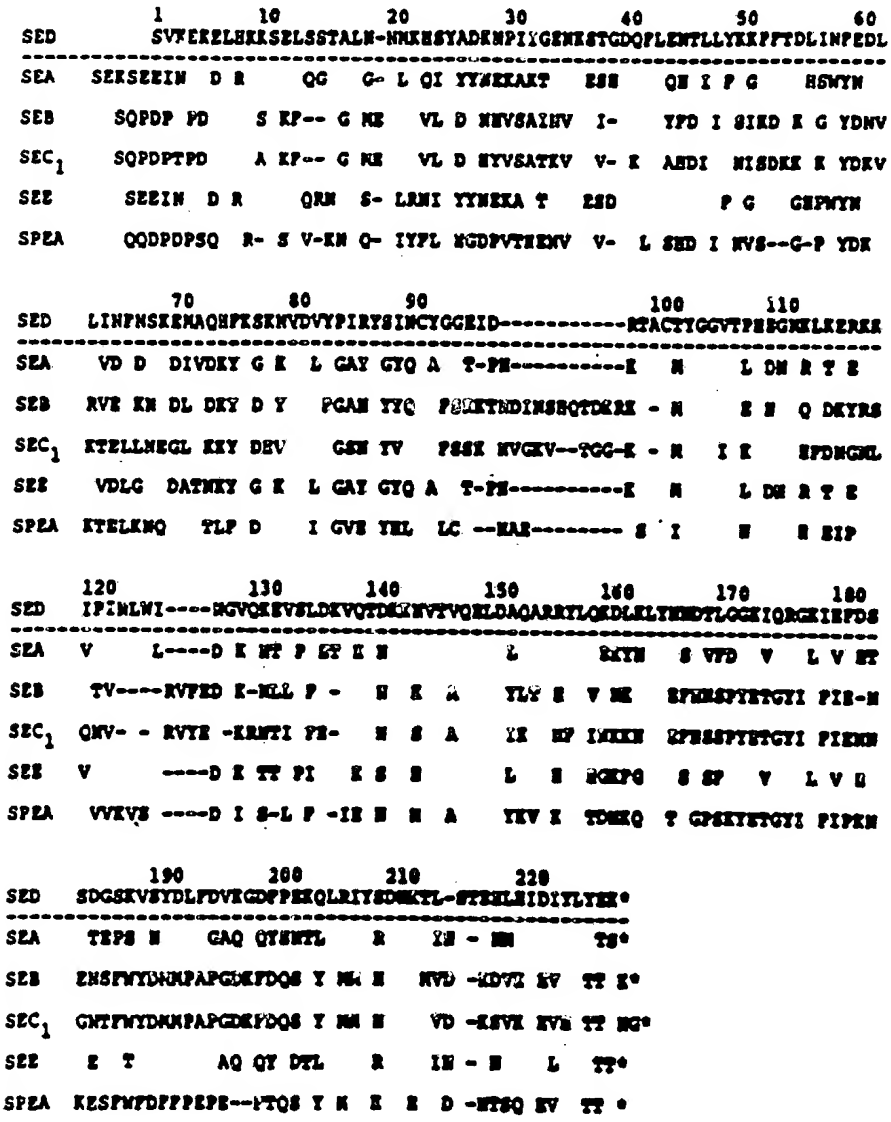
The Staphylococcal enterotoxins include A, B, C_{1-3}, D, and E; B being of particular interest. Each of the Staphylococcal enterotoxins have a binding site which binds to at least one, but fewer than all, of the variable regions of the T-cell receptors of rodent, particularly mouse, and primate, particularly human, T-cell receptor variable regions, more particularly the β-subunit. In addition, these peptide portions of the Staphylococcal enterotoxin may also be used to induce the down and up regulation of different surface membrane proteins. It is found that expression of the CD3-Ti antigen receptor complex is down regulated following exposure to the enterotoxin. In addition, CD25 (IL-2 receptor) is up regulated by the presence of the subject peptide.

The enterotoxin fragments will generally be of at least 8 amino acids, more usually at least about 12 amino acids, and may be 20 amino acids or more, up to and including the entire enterotoxin. Preferably, fragments will be used which substantially reduce the enterotoxin toxin activity, while maintaining the ability to induce T-cell non-responsiveness. Derivatives may be methylated, acylated, e.g. acetylated, conjugated to immunogens or analogues having addition or replacement with an unnatural amino acid.

The sequences of the enterotoxins as described by James C. and Khan S. J. Bacteriol. 166: 29-33 (1986); Couch, J. L. et al., J. Bacherol, 170: 2954-2960 (1988); Betty, M. and Mekalamos, J., J. Bacherol, 170,

34-41 (1988); Boach, G. A. and Schlievert, P., Mot. Gen. Genet., 209: 15-20 (1987); Schmidt, J. and Spero, L., J. Bio. Chem., 258: 6300-6306 (1983); see particularly, Iandola, Amer. Rev. Microbiology., 43: 375-402 (1982); are as follows:

TABLE



Alignment of the protein sequences of the Staphylococcal enterotoxins and Streptococcal pyrogenic exotoxin A. Single-letter amino acid codes are used to indicate differences from the index sequence, entero-

toxin D. Dashes indicate gaps introduced to produce the optimum alignment of amino acids.

Of particular interest is a sequence of at least eight amino acids, preferably at least about 12 amino acids, in the sequence from about 1 to 100, preferably 1 to 40, more preferably 1 to 27, of the enterotoxins, or sequence having at least about 70% hemology, preferably at least about 80% hemology between two or more enterotoxins, particularly including SEB.

The subject compositions may be used in the suppression of rejection reaction in transplantation, to treat autoimmune diseases, rhinitis, exemia, asthma, harmful responses to infectious agents, such as tuberculoid leprosy and to treat allergic responses. In the case of suppressing the rejection reaction, transplantation of various organs may be involved, such as bone marrow, skin, kidney, heart, and liver.

The subject compositions may be administered by any convenient procedures particularly parenterally, such as intravascular, e.g. intravenous, intraperitoneal, intramuscular, and subcutaneous. Depending upon the purpose for which the subject compositions are administered, the subject compositions may be administered prior to, simultaneously with, or subsequent to the occurrence of the condition for which the subject compositions are administered.

The subject compositions may be used as individual compounds or combinations of compounds. Depending upon the host, the range of the subject composition in providing immunosuppression, and the degree of immunosuppression desired, one or more compounds may be involved. Thus, one or more of the subject compositions may be employed, by themselves or in conjunction with other agents, such as antibodies to T-cell markers e.g. CD^4, immunosuppression agents, e.g. cyclosporin, FK506, methotrexate cyclophosphamide etc., antihistamines, corticosteroids, antiinflammatories, non-stimulatory MHC, competitive binding proteins,

theophylline, β2-agonists, etc. The combinations may be used for the survival of transplants and the therapy of autoimmune and allergic disease.

The frequency and duration of administration will depend upon the particular condition being treated, the condition of the patient, the degree to which immunosuppression is to be maintained, the effectiveness and lifetime of the particular compounds employed, and the like. Dosage will vary widely based on the above factors, but will generally be in the range of about 0.5-50μg of the subject compounds per 1 kg of body weight.

The subject compositions will normally be administered in a pharmaceutically acceptable carrier such as physiological saline, phosphate buffered saline, or other buffer solution. The concentration of the subject compositions in such liquid carrier will generally range from about 0.05 to 2mg/ml. Other additives which may be included in the medium include stabilizers, preservatives, osmoticums, etc., which are conventional in the administration of drugs and do not interfere with the activity of the subject compositions.

The subject compositions may be prepared in a variety of ways. For peptides under about 30 amino acids, the subject compositions may be synthesized in accordance with conventional techniques or by using an automatic apparatus. Conveniently, protected amino acids are employed with a support, where the amino acids are added sequentially to the growing chain. When the chain is completed, the peptide may then be removed from the support and the protective groups also removed. By using synthetic techniques, various natural or unnatural amino acids may be employed to provide for enhanced stability of the product from proteolysis, allow for linkage to carriers, and the like.

Alternatively, for peptides of greater than about 30 amino acids, particularly above 60 amino acids, recombinant techniques may be employed where a DNA

sequence encoding the desired amino acid sequence may be introduced into an expression vector and then transformed into an appropriate host for expression. Expression vectors may include a signal sequence which allows for secretion of the product from the host. A wide variety of expression vectors are commercially available or have been described in the literature, which expression vectors may be used with advantage. Hosts may be prokaryotic or eukaryotic, particularly prokaryotic.

In some instances, it may be desirable to mutate one or more amino acids of the peptide, usually not more than about 10% of the amino acids present in the peptide, more usually not more than about 5%. Generally, not more than about 1 to 5 amino acids, more usually not more than about 1 to 3 amino acids will be involved. The substitutions may be conservative or non-conservative, where conservative intends that the same general conformation, size and polarity is involved. That is, charged molecules will normally not be substituted for hydrocarbon, while polar molecules depending upon their conformation may be substituted for either charged or non-polar molecules, e.g. isoleucine for glutamine, asparagine for lysine or aspartic acid.

The subject compositions may also be used in vitro, where a mixture of T-cells are involved for determining the presence of the analogous variable regions to which the subject compositions bind. For example, by carrying out a control run in the absence of a subject composition and a sample run, such as a mixed lymphocyte reaction, a reduction in the amount of T-cell responsiveness in the sample as compared to the control would indicate the presence of the complementary variable region(s). The subject compounds may also be used to determine whether cells are capable of expressing CD28 by combining the cells with the subject composition and assaying for the presence of CD28 with an appropriate antibcdy. The subject compositions may

also be used to determine regulation of expression of other receptors.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

1. The effect of Staphylococcal enterotoxin B (SEB) on the proliferative response of HA1.7.

The isolation and characterization of the HLA-DR1 restricted T-cell clone HA1.7 that recognizes the carboxyl terminus of the HA-1 influenza virus hemoagglutinin (HA, residues 307-319) is described in Lamb et al., Nature 300: 66-69 (1982). Lymphoblasts activated with immunochemically purified HA were cloned by limiting dilution in the presence of irradiated autologous peripheral blood mononuclear leucocytes (PBMC), HA and Interleukin 2 (IL-2) in RPMI-1640 (Gibco, Grand Island, New York) supplemented with 10% human A+ serum.

T-cells of HA1.7 (10^5/ml) were cultured together with increasing concentrations of Staphylococcal enterotoxin B ($5x10^{-4}$ to 50μg/ml; SEB, Sigma, St. Louis, Missouri) alone, IL-2 (10% $^{v}/_{v}$ Lymphocult T, Biotest Frankfurt, Federal Republic of Germany) and SEB in the presence of histocompatible irradiated PBMCs (1.25×10^5/ml). Additionally, cloned T-cells (10^5/ml) were incubated in medium alone or with SEB for 16 hours in the absence of accessory cells. After washing, the T-cells were cultured or mitomycin C treated murine fibroblasts expressing HLA-DR1 (10^5/ml) pulsed with HA 307-319 (1 μg/ml).

After 60 hours of incubation, tritiated methyl thymidine ([^{3}H]TdR; 1 μCi/ml; Amersham International, Arlington Heights, Illinois) was added and the cultures harvested onto glass fiber filters 8-16 hours later. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as mean counts per minute (CPM)

for triplicated cultures. In all cases the standard error of the mean <20%.

HA1.7 when cultured either alone or in the presence of histocompatible irradiated PBMCs proliferated to Staphylococcal enterotoxin B (SEB) over a broad concentration range, but weakly to the response to the natural ligand. Responsiveness of the T-cells to exogenous IL-2 was marginally enhanced in the presence of SEB. SEB was able to modulate antigen recognition by the cloned T-cells in a dose dependent manner, such that the T-cells were unable to respond to an immunogenic challenge of the appropriate ligand at concentrations of SEB <0.05 μg/ml. The failure to respond to antigen was not due to cytolysis since IL-2 responsiveness was maintained and at certain concentrations of SEB enhanced. This phenomenon of T-cell non-responsiveness is similar to that induced by free antigen in peptidic form (Lamb et al., (1983) supra) or antigen presented by chemically modified accessory cells (Jenkins and Schwartz, J. Exp. Med. 165: 302-319 (1987)). This suggests SEB is able to functionally inactivate human T-cells in a manner similar to that reported for specific tolerance to Mls-1a in adult Mls-1b mice (Rammensee et al., (1989) supra).

Response, cpm [^{3}H]-TdR*

SEB conc.	HA1.7 alone	HA1.7 + 10% IL-2	HA1.7 + APC	HA1.7 + APC + HA307-319
0	650	4300	900	28689
0.0005 g/ml	42	6047	640	31141
0.005	32	5786	597	28701
0.05	60	5524	708	31959
0.5	2764	8905	3214	3021
5	4105	7613	6585	2353
50	2708	4848	4358	2169

*cpm [^{3}H]-thymidine deoxyuribhose incorporation

2. Functional inactivation of T-cell clone HA1.7 following exposure to SE is associated with modulation of CD3 and CD25 expression.

T-cells of HA1.7 were incubated for 16 hours with SEB, HA (307-319), residues 36-60 of Der p II (from the house dust mite) at the following concentrations: SEB 5x10-4 - 50μg/ml at 10 fold increments; HA 307-319; 50 μg/ml; Der p II 56-60, 50 μg/ml. Control cultures contained insolubilized anti-CD3 (12 μg/ml) and IL-2 or medium alone. The cells were washed and stained directly with saturating concentrations of fluorescein conjugated murine monoclonal antibodies anti-Leu-4 (CD3), anti-IL-2 receptor (CD 25) or mouse IgG1-FITC control (Becton Dickinson, Mountain View, California). Only viable cells, identified by their ability to exclude propidium iodide, were analyzed by flow cytometry using a FACScan (Becton Dickinson). The cell population was analyzed by gating on the volume and light scatter characteristics.

Aliquots of the T-cells (10^5ml) from each group of treatments were assayed for their ability to respond to an immunogenic challenge of HA 307-319 and accessory cells (mitomycin C treated murine fibroblast expressing HLA-DR1) or accessory cells alone as described above.

Expression of the CD3-Ti antigen receptor complex was down regulated following exposure to superantigen (<0.5 μg/ml), which correlated with the failure of the T-cells to proliferate to specific peptide. Incubation of the T-cells with HA 307-319 at supraimmunogenic concentrations, but not the control peptide of a relevant specificity, also reduced membrane levels of CD3-Ti. Although activation of the T-cells with insolubilized anti-CD3 antibody and IL-2 modulated CD3 from the cell surface, the kinetics of recovery of antigen specific responsiveness was more rapid than that of either SEB or peptide induced anergy which was till present at 5 days. Concomitant with the

modulation of CD3-Ti, expression of CD25 (IL-2R) was up regulated in SEB and HA peptide induced non-responsiveness as well as activation. This suggests that the T-cell anergy induced by SEB is associated with the down regulation of the antigen receptor that is paralleled by enhancement of CD25 expression.

3. The effect of SEB and peptide induced non-responsiveness on the phenotype of T-cell clone HA1.7.

T-cells of clone HA1.7 were incubated in medium alone, anti-CD3 antibody and IL-2, or with SEB and HA peptide. The latter two treatments were under conditions that induce non-responsiveness. The cells were washed and stained directly with saturating concentrations of fluorescein conjugated the murine monoclonal antibodies anti-Leu 5b (CD-2), anti-Leu 4 (CD3), anti-Leu 3a (CD-4), anti-IL-2 receptor (CD25), using a mouse IgG1 FITC control, or indirectly with anti-CD28 (9.3, Hansen et al., Immunogenetics 10: 247-252 (1980)). Flow cytometric analysis was performed as described above.

No co-modulation of CD4 with CD3 was observed suggesting that CD4 is not part of the antigen recognition complex of this T-cell clone (Saizawa et al., Nature 328: 260-263 (1987)). However, the expression of CD2 both in activation and the induction of anergy is reciprocal to that of CD3. This suggests that the two populations of CD2 are modulated independently and may have different functional roles in the regulation of T-cell activation.

The levels of CD28, a membrane glycoprotein associated with an alternative pathway of T-cell activation independent of antigen recognition by the CD3-TI complex (Gmunder and Lipsky, Eur. J. Immunol. 142: 153-160 (1984); Martin et al., J. Immunol. 136: 3282-3287 (1986) was marginally down regulated in both SEB and HA peptide induced non-responsiveness.

Enhanced expression of CD28 was observed in anti-CD3 activated T-cells.

It is evident from the above results, that enterotoxin may be employed to provide for substantial reduction in immune responsiveness, in those situations where reduction in immune responsiveness is desired. Furthermore, the enterotoxin may be directed to specific variable regions of the T-cell receptor, so that only a portion of the immune response is modulated. Thus, the subject compositions may be used in the treatment of a wide variety of diseases, as well as in various diagnostic situations to determine the nature of the T-cell receptors present in a sample, the ability of the T-cells to respond to the subject compositions, the ability of T-cells to mount an immune response, and the like.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for reducing the immune response of a lymphocytic composition comprising T-cells, said method comprising:

adding to said lymphocytic composition an amount effective to reduce the immune response of a Staphylococcal enterotoxin, active fragment or dermative thereof.

2. A method according to Claim 1, wherein said enterotoxin is Staphylococcal enterotoxin A, B or D.

3. A method according to claim 2 wherein said enterotoxin B.

4. A method according to Claim 1, wherein the whole enterotoxin is employed.

5. A method for determining the responsiveness of T-cells to immunomodulation by a Staphylococcal enterotoxin, said method comprising:

combining said T-cells with said enterotoxin; and

determining the change in the level of expression of said T-cells of CD3 and/or CD25.

6. A method according to Claim 5, wherein said enterotoxin is enterotoxin, A, B or D.

7. A method according to Claim 6, wherein said enterotoxin is B.

8. A method for reducing the immune response in a mammalian host, said method comprising:

administering to said host an amount effective to reduce the immune response of a Staphylococcal enterotoxin, active fragment or derivative thereof.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01028

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/02; C12N 5/02; G01N 33/567

US CL: 424/92 435/240.2, 7.24;436/503

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	424/92; 435/240.2, 7/24; 436/503

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Journal of Experimental Medicine, Volume 165, issued February 1987, Marc K. Jenkins et al. "Antigen Presentation by chemically modified splenocytes induces antigenspecific T cell unresponsiveness in vitro and in VIVO" pages 302-319, see entire article.	1-8
Y	Nature, Volume 339, issued 15 June 1989. Rammensee et al, "Clonal anergy induced in mature VB6+ T Lymphocytes on immunizing M/S^b mice with $M15\text{-}1^a$ expressing cells", pages 541-544, see entire article.	1-8
Y	Cell, Volume 56, issued 13 January 1989, J. White et al, "The VB-Specific Superantigen Staphylococcal Enterotoxin B: Stimulation of Nature T cells and clonal Deletion in Neonatal Mice". pages 27-35, see entire article.	1-8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report [14]
10 May 1991	20 JUN 1991
International Searching Authority	Signature of Authorized Officer [15]
ISA/US	Hazel Sidberry

Form PCT/ISA/210 (second sheet) (July 1989)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, volume 303, issued 16 June 1983, E.D. Zaunders et al, "Tolerance of T-Cells is associated with membrane antigen changes", pages 625-627, see entire article.	1-8
Y	Science, volume 244, issued 19 May 1989, J. Kappler et al, "VB-Specific Stimulation of Human T cells by Staphylococcal Toxins," pages 811-813, see entire article.	1-8
Y	The Journal of Immunology, volume 140, No. 8, issued 15 April 1988, Roland Carisson et al, "Binding of Staphylococcal Enterotoxin A to Accessory cells is a Requirement for its ability to activate Human T Cells" pages 2484-2488, see entire article.	1-8
Y	The Journal of Immunology, volume 105, No. 6, issued December 1970, D.L. Peavy et al, "The mitogenic Effects of Endotoxin and Staphylococcal enterotoxin B on Mouse Spleen cells and Human Peripheral Lymophocytes", pages 1453-1458, see entire document.	1-8

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [1]

This International Searching Authority found multiple inventions in this international application as follows:

See telephone ~~practice~~ memorandum form

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: telephone practice

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without the request for additional fees, this Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT Telephone Memorandum

I. Claims 1-4 and 8 are drawn to a method for reducing the immune response of a Lymphocytic Composition comprising T-cells by adding Staphylococcal enterotoxin. Classified in classes 424 and 435 subclasses 92 and 240.2.

II. Claims 5-7 are drawn to a second method a method for determining the responsivenes of T-cells immunomodulation by Staphylococcal enterotoxin classified in classes 435 and 436 subclasses 7.24 an 503.

The claims of these two groups are directed to different inventions which are not linked as to form a single inventive concept. The inventions differ in method steps and detection mechanisms.